Preliminary evaluation of *in vitro* cytotoxic effect of non-thermal plasma on fibroblast cells

Waraluk Yarangsee[1,2], Pornsiri Pitchakarn[3], Supakit Khacha-ananda[4,*]

[1] Master's Degree in Toxicology, Department of Forensic Medicine, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand; waraluk_ya@cmu.ac.th
[2] Graduate Shool, Chiang Mai University, Chiang Mai 50200, Thailand
[3] Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand; pornsiri.p@cmu.ac.th
[4] Department of Forensic Medicine, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand; supakit.kh@cmu.ac.th
* Correspondence: supakit.kh@cmu.ac.th; Tel.: +6689-5537211

Abstract: Non-thermal plasma (NTP) is an ionized gas generated by the ionization between gas molecules (carrier gas) and an electric field under atmospheric pressure. The various kinds of reactive oxygen/nitrogen species (ROS/RNS) were suddenly generated during gas ionization. An appropriate reactive species exerted beneficial effects in medicine, therefore, NTP technology has been recommended and developed to use in medical treatments. However, the excesses of reactive species have been reported to disturb cellular physiology such as cell death, neurodegeneration, and cancer. Hence, this study aimed to investigate the cytotoxic effect of non-thermal plasma (NTP) on cell viability and proliferation of murine embryonic fibroblast cell lines (3T3-L1 cells). The MTT and colony formation assay were performed to demonstrate the cytotoxic and proliferative activity of NTP-exposed cells. Our result showed that the percentage of the cell viability and colony formation was significantly decreased after treatment of the cells with all doses of NTP. The highest cytotoxicity was observed in the cells which were exposed with NTP at intensity 10 pluses under air flow rate 11 L/min. In addition, the greatest toxicity to inhibit cell proliferation was found in the cells which were exposed with NTP at intensity 7 and 10 pluses under air flow rate 11 L/min. In conclusion, the results suggested that NTP treatment affected cell viability and cell proliferation of 3T3-L1 cell.

Keywords: Non-thermal plasma/carrier gas/ reactive oxygen/nitrogen species (ROS/RNS)

1. Introduction

Plasma, the fourth state of the matter, is an ionized gas generated by the subjecting gas into an electromagnetic field [1]. It can be classified into two types; thermal and non-thermal plasma according to relative temperature of electrons, ions and neutrals [2]. The non-thermal plasma (NTP) or cold plasma is generated by the ionization of carrier gases such as helium (He), argon (Ar), nitrogen (N_2), ambient air or mixture of inert gases under high voltage of electromagnetic field. During this process, many chemical substances especially reactive oxygen species (ROS) for example superoxide ($\bullet O^{2-}$), hydrogen peroxide (H_2O_2), hydroxyl ($\bullet OH$), and ozone (O_3) as well as reactive nitrogen species (RNS) for example nitric oxide (NO), nitrate (NO^{2-}) and nitrite (NO^{3-}) were dominantly produced [3-5]. These reactive molecules subsequently interacted with cell membranes and accumulated in the cell to induce cellular responses [6]. Depending on utilize working gas, the difference in working gas between argon, helium, or ambient air has been reported to

influence the chemical active species of plasma [5, 7, 8]. The largest amounts of ROS were produced by argon and helium plasma, whereas the highest level of RNS production was also produced by ambient air plasma [8]. The ROS and RNS have long been known to be key signaling molecules on physiological processes in several cell types [9, 10]. The valuable aspects of reactive species were related to control cellular redox status and involved in signaling cascade [11]. Therefore, NTP-produced reactive species have been suggested to mediate several biological responses to inhibit microorganisms such as bacteria, fungi, and viruses, decrease inflammation, and accelerate wound healing process [1, 12-14]. However, the excess use of NTP causing high amounts of reactive species production affected the transformation of some cellular biomolecules such as lipid, protein, carbohydrate, and genetic materials [10, 15]. The deleterious effects of reactive species on genetic materials were well characterized as a risk factor for mutation and cancer development [1, 15]. As a result, these side effects of plasma technology should be monitored prior to its application in clinical therapy. Moreover, the characteristics of innovative medical devices such as biocompatibility and biological safety need to be demonstrated before launching new products according to the International Organization for Standardization 10993 (ISO 10993) guideline [16, 17]. The *in vitro* cytotoxicity test should be performed to determine the fitness of a device for human use and provide safety of a new medicine device without any potentially harmful physiological effects. Therefore, the aim of this study is to evaluate the cytotoxic and proliferative activity of fibroblast cell lines after exposure with non-thermal plasma (NTP).

2. Materials and Methods

2.1. Non-thermal plasma device

The NTP device in this study was supported by the Plasma and Beam Physics Research Facility, Faculty of Science, Chiang Mai University, Thailand. The NTP is established by a coaxial DC pulse-adjusted kHz forced atmospheric pressure air plasma jet at difference flow rate (L/min). Typical ambient air as a carrier gas at temperature between 27.5±0.95°C and 61±6.0% relative humidity (RH) was passed over the coaxial gap channel around the insulator rod by a DC air pump. The plasma jet device included a 1.2-mm-diameter 304L powered electrode and a 9-mm-inner-diameter a grounded electrode with a 1-mm-diameter outlet nozzle. The tip of the high-voltage (HV) electrode was located 1.0 mm above the grounded electrode. The powered electrode was forced by a series of HV pulses in a burst mode [3].

2.2. Cell lines and culture

The murine embryonic fibroblast cell lines or 3T3-L1 (ATCC® CL-173™) were kindly obtained from Asst. Prof. Dr. Pornsiri Pitchakarn, Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco™, UK.) supplemented with 10**%** heat-inactivated fetal bovine serum (Hyclone, UT, USA), 100 µg/ml of streptomycin, and 100 U/ml of penicillin (Capricorn Scientific, Germany)**.** The cells were maintained at 37 ˚C in a humidified atmosphere 95**%** containing 5**%** CO_2. Prior experiment, the cells were harvested by trypsin-EDTA solution (Hyclone, UT, USA) and washed with steriled phosphate buffer saline (PBS). The cell viability was determined by Trypan blue exclusion assay and adjusted to a desire cell number.

2.3. Assessment of cytotoxicity effect

To investigate the cytotoxic effect of NTP on 3T3-L1 cells, the cells ($2x10^5$ cells) were seeded on a 12-well tissue culture plate (Costar®, CA, USA) at 37 ˚C in a humidified atmosphere of 95**%** containing 5**%** CO_2 for 24 h. After that, the cells were directly exposed with different dose of NTP according to previous publication [18]:

Cell control:	Cells were maintained in DMEM without NTP exposure.
Vehicle control:	Cells were exposed with different flow rate of ambient air (3, 5, 7, 9, 11 L/min) as carrier gas without NTP generation.
Experiment group:	Cells were exposed with different NTP intensity (4, 7 and 10 pluses) with ambient air as carrier gas.

After NTP exposure, the cells were subsequently incubated at 37 ˚C in a humidified atmosphere of 95**%** containing 5**%** CO_2 for 24 h, The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solutions at concentration of 2 mg/ml (PanReac AppliChem ITW Reagents, Germany) were then added to each well and the plate was continuously incubated at 37 ˚C for 4 h. After incubation, the supernatants were discarded, and dimethyl sulfoxide (DMSO) was added into each well to dissolve formazan crystal. Finally, the absorbance was measured at a wavelength of 570 and 630 nm by microplate reader (*BioTek™ Synergy™ H4 Hybrid*, VT, USA). The cytotoxic effect was expressed as a percentage of cell viability and calculated as follows:

$$\% \text{ cell viability} = \frac{\text{absorbance of experiment group}}{\text{absorbance of cell control}} \text{ x100}$$

2.4. Assessment of cell proliferation

To determine the effect of NTP on 3T3-L1 cell proliferation, the colony formation assay was performed. The cells ($2x10^5$ cells) were seed on a 12-well tissue culture plate (Costar®, CA, USA) at 37 ˚C in a humidified atmosphere of 95**%** containing 5**%** CO_2 for 24 h. After incubation, the cells were directly exposed with different dose according to previous experiment for 30 s. The treated cells were subsequently harvested and stained with trypan blue to evaluate cell viability. Five-hundred viable cells were transferred to a 6-well tissue culture plate and the plate were incubated at 37 ˚C in a humidified atmosphere of 95**%** containing 5**%** CO_2 for 7 days. At the end of incubation, the colonies were fixed and washed with sterilized PBS. The colonies were then stained with 0**.**5**%** w/v of crystal violet for 20 mins. The number of colonies containing at least 50 cells were observed under inverted microscopy (Olympus CK40, Japan), and the percentage of colony formation was calculated as follows:

$$\% \text{ colony formation} = \frac{\text{colony number of experiment group}}{\text{colony number of cell control}} \text{ x100}$$

2.5. Data analysis

Data were expressed as mean ± standard deviation of three independent determinations in three replicates for each experiment. The percentage of cell viability and colony formation were compared between cell control, vehicle control, and experimental group. The independent sample t-test was carried out to indicate a statistically significant difference with p-value <0.05**.**

3. Results

3.1. The suppression of cell viability by NTP

To demonstrate the cell viability of 3T3-L1 cells after treatment with NTP, the cells were exposed with different dose of NTP for 30 s and the cell viability was done by MTT assay. The percentage of cell viability in cell control was varied between 100±3.06 to 100±5.74. The cells exposed with ambient air as a carrier gas without NTP generation (vehicle control) gave the percentage of cell viability between 98.35±4.27 to 102.48±8.01. The percentage of cell viability was ranged between 75.61±6.98 to 97.83±3.95 for NTP-exposed

cell ranging from 4 to 10 pluses. At each plasma intensity, the percentage of cell viability was a significantly decrease in dose-dependent manner after exposure the cells with different air flow rate **(**Fig**.** 1). Moreover, the lowest cell viability considering as the most cytotoxicity was observed in the cells exposed with NTP at intensity 10 pluses with air flow rate 11 L/min.

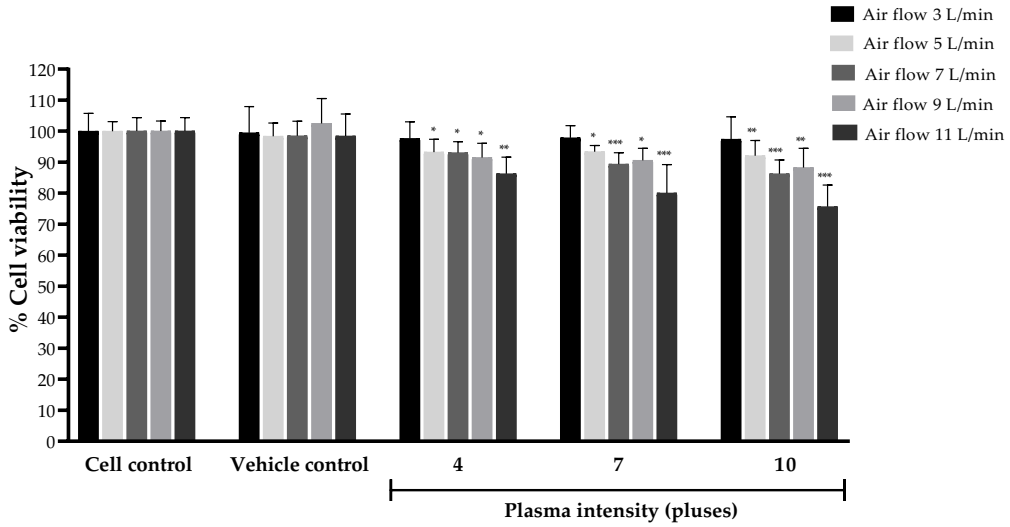
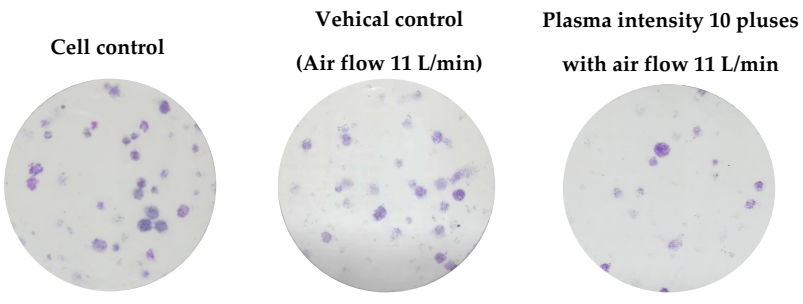


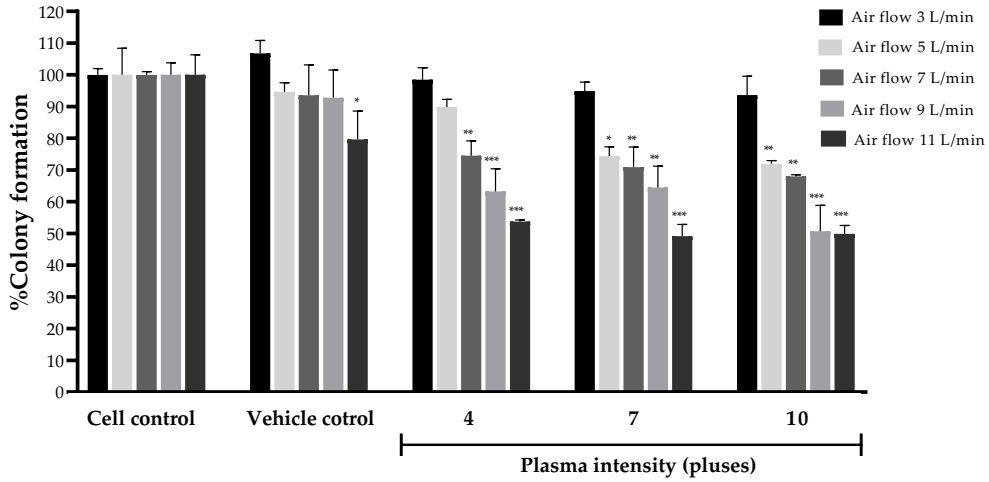
Figure 1. The percentage of cell viability on 3T3-L1 cells after exposure the cells with different air flow and NTP intensity was determined by MTT assay. Data represent mean of three independent experiments with three technical replicates each ± standard deviation (SD). Statistical analysis was performed by independent sample t-test with statistical significance comparing with cell control, $p < 0.05$ (*), $p < 0.001$ (**), $p < 0.0001$ (***).

3.2. The reduction of cell proliferation by NTP

To demonstrate the cell proliferation of 3T3-L1 after exposure the cells with different intensity of NTP, the colony formation assay was investigated. As indicated in figure 2, cell and vehicle control had the percentage of colony formation varied from 100±1.03 to 100±8.36 and 79.65±8.90 to 106.74±4.11, respectively. However, the percentage of colony formation in vehicle control was remarkably decreased at air flow rate 11 L/min. Similarly, the reduction of cell proliferation in NTP-exposed cells with different air flow rate (experiment group) was a dose-dependent of NTP intensity. The percentage of colony formation in experiment group was gave at 49.14±3.72 to 98.48±3.78 and remarkably lower than cell control. Furthermore, the most effective to decrease the colony formation was NTP intensity at 7 and 10 pluses with the highest air flow rate.



(a)



(b)

Figure 2. The investigation of cell proliferation after exposure the cells with NTP was determined by colony formation assay. (a) The colony formation of NTP-exposed cells was stained with 0.5% w/v of crystal violet and observed under light microscope (40X). (b) The percentage of a colony formation on 3T3-L1 cells exposed with different air flow and NTP intensity was determined by colony formation assay. Data represent mean of three independent experiments with three technical replicates each ± standard deviation (SD). Statistical analysis was performed by independent sample t-test with statistical significance comparing with cell control, $p < 0.05$ (*), $p < 0.001$ (**), $p < 0.0001$ (***).

4. Discussion

Although NTP has been previously reported to have the beneficial medical application, the characteristic of NTP producing the high amounts of reactive substances has been considered to lessen severe side effects. NTP mediated several biological responses such as antimicrobial, anticancer, anti-inflammatory activity through the production of reactive species especially ROS/RNS [1, 3]. However, high amounts of reactive species released during plasma generation were to generate a cytotoxic effect and induce cell death through disruption of redox status [19]. Therefore, we aimed to study the effect of NTP generated from ambient air as carrier gas on the murine embryonic fibroblast cell lines (3T3-L1 cells) for further clinical applications on human skin. These cell lines were suggested for basic toxicity screening since they maintain all basal cellular functions and give a more reproducible way than primary cells. According to Fröhlich, 2018, the NIH/3T3 cells derived from mouse embryonal fibroblasts were recommended to use as the physiologically relevant fibroblast models[20]. Moreover, these cell lines were mostly used as a skin model for evaluating the effect of various chemical on cytotoxicity, cell proliferation, anti-inflammation, and wound healing [22-25].

Our study use showed that cell viability and cell proliferation was remarkably decrease in a dose-dependent manner after exposure the cells with different intensity of NTP. The possible explanation could be explained that the high amounts of ROS/RNS during plasma generation diffused across the cell membrane to accumulate in the cell [10, 21-23]. The cellular macromolecules (lipid, protein, and genetic material) and organelles (mitochondrial, endoplasmic reticulum, and lysosome) were subsequently destroyed [23]. Previous publications have been demonstrated the effect of a dielectric barrier discharge plasma (DBD) on cell membrane structure. The significant increase of malondialdehyde (MDA) which is a by-product from lipid peroxidation of cell membrane was observed in NTP-exposed breast epithelial cells [24]. The exposure of the keratinocyte cells with NTP which was produced from helium-oxygen gas demonstrated the abnormal morphology and depolarization of cell membrane. The plasma membrane integrity by the detection of inner mitochondrial membrane potential ($\Delta\psi m$) was obviously decreased in the human periodontal ligament (PDL) cells exposed with NTP [25, 26]. In addition, the findings of

cell membrane damage, cytoplasm leakage, mitochondrial, and ER swelling were observed in the treatment of low-temperature argon plasma on murine fibroblast cell lines [27]. The subsequent effect of mitochondrial membrane dysfunction also triggered the release of proapoptotic proteins into cytoplasm leading to activate cell death through apoptosis pathway [23, 28-30]. The apoptotic cell death through intracellular ROS production was significantly stimulated in the incubation of fibroblast cells with argon plasma-activated medium [31]. Likewise, the previous study of Lunov *et al*., 2014 showed that the percentage of apoptotic cell death was dramatically increased after exposure of fibroblast cells with different types of plasma (air and helium). Previous result implied that this phenomenon could be resulted from the production of superoxide radical during plasma generation [28]. However, other assumption for cell death induced by NTP generated from argon gas was probably based on the failure of DNA repairing processes [15, 32, 33]. Although some intensities of NTP showed the less cytotoxic effect on fibroblast cells, the inhibition of cell proliferation was observed in the numerous doses of NTP without cytotoxic effect. Our study found that the cell proliferation was significantly decreased in NTP-exposed cells. NTP intensity at 7 and 10 pluses with the highest air flow rate (11 L/mins) revealed the most effect to decrease the cell proliferation representing as the reduced number of colony formation. It was believed that the inhibition of cell proliferation resulted from the cell cycle arrest. According to the previous studies, the cell cycle at G2/M phase was blocked after direct treatment of human keratinocytes (HaCaT cells) with DBD plasma and plasma jet (pure oxygen and argon as carrier gas) [34-36]. Finally, the cytotoxic and proliferative effects of NTP generated from ambient air on fibroblast cell lines was established in our study since the use of ambient air as carrier gas for NTP production has less been performed as described above.

The inconsistent results between MTT and colony formation assay was probably resulted from the principle of test. The lack of cell-cell and cell-matrix adhesion properties was an important factor affecting colony formation results. The single colony of the cells was plated on the well and observe the expansion of colony. These properties also promote the activation of clonogenicity (the single cell property to clone itself and grow into a full colony of cloned cells) which can be observed by colony formation assay[37, 38]. However, the MTT assay is based on the detection of metabolic activity in mitochondria which can be classified into viable and non-viable cells without the clonogenic property since the cells were grown until almost 100% confluent on the plate. Therefore, it was probable that clonogenic property and metabolic status of the cells are not necessarily the parallel phenomena. The prematurely senescent cells which were metabolically active might not establish colony.

5. Conclusions

In this work, we summarized that the reduction of cell viability and proliferation was a dose-dependent manner after exposure murine embryonic fibroblast cell lines (3T3-L1 cells) with non-thermal plasma which was generated from ambient air.

Supplementary Materials: None.

Author Contributions: Conceptualization, W.Y., P.P., and S.K.; methodology, W.Y., P.P., and S.K.; formal analysis, W.Y.; data curation, W.Y., P.P., and S.K.; writing—original draft preparation, W.Y. and S.K.; writing—review and editing, W.Y., P.P., and S.K.; visualization, S.K.; supervision, S.K.; project administration, S.K.; funding acquisition, S.K. All authors have read and agreed to the published version of the manuscript.

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